Molecular Modification of Anticholinergics as Probes for Muscarinic Receptors. 2. Amino Esters of α-Methyltropic Acid[1]

Matthias C. Lu,*† Lisa B. Shih,† Hwan S. Jae,† James E. Gearien,† and Emmanuel B. Thompson‡

Department of Medicinal Chemistry and Pharmacognosy and Department of Pharmacodynamics, College of Pharmacy, The University of Illinois at Chicago, Chicago, Illinois 60680. *Received November 25, 1985*

As a continuation of our goals to study molecular probes for muscarinic cholinergic receptors, a series of 3-substituted 2-methyl-2-phenylpropanoates with the general structure of $C_6H_5C(CH_2X)(CH_3)COOCH_2CH_2NEt_2$ where X = OH, OTs, F, Cl, Br, I, and OAc were prepared and their antispasmodic activities examined on isolated rat ileum preparations. Structure–activity relationship studies with these compounds provide further evidence suggesting that binding of an aromatic moiety in a specific location within the hydrophobic region of the receptor is important for anticholinergic potency. A nucleophilic displacement of chloride by "naked" fluoride under mild conditions is also reported.

While it may be assumed that atropine-like anticholinergics, because of their structural similarity to acetylcholine (ACh), may bind in a similar manner to muscarinic receptors, portions of their structure would extend to areas not occupied by ACh or other muscarinic agonists.[2,3] Studies in our laboratory have utilized muscarinic antagonists as probes for exploring the topographical areas of the muscarinic receptor that contribute to the binding of anticholinergic agents. As a continuation of this work, a series of (diethylamino)ethyl esters of 3-substituted 2-methyl-2-phenylpropanoic acid with the general structure of $C_6H_5C(CH_2X)(CH_3)COOCH_2CH_2NEt_2$ (see Table I for structures) were prepared and their antispasmodic activities evaluated on isolated rat ileum preparations. These compounds were prepared as structural probes to ascertain the possible existence of a hydrogen-bonding subsite previously mentioned in the literature.[2,4] The tosyloxy analogues of α-methyltropate, being more stable than the tosyloxy analogues of tropate,[5] were examined for their ability to alkylate the hydrogen-bonding subsite.

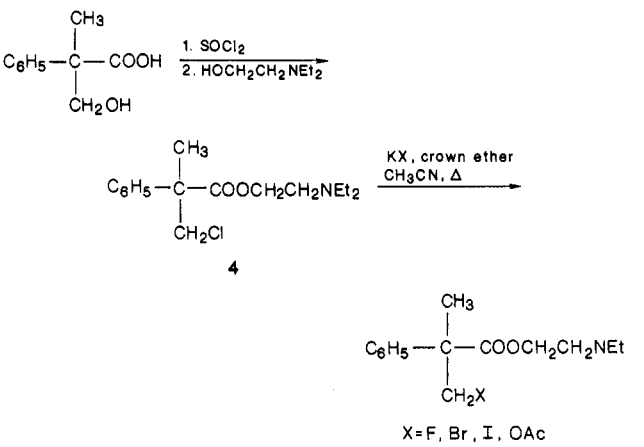
Moreover, we describe the synthetic utilization of crown ether catalyzed direct nucleophilic displacement of halide by "naked" anions. This procedure provides an opportunity for the synthesis of atropine-like anticholinergics containing fluorine under mild conditions and with excellent yield.

Chemistry

The method of Vechhi and Melone[6] with minor modification of the last hydrolysis step was used for the preparation of α-methyltropic acid (8). This compound was successfully resolved into its optically active isomers. Both the racemic acid and its optical isomers were easily converted to the desired amino esters (method A) and their tosyloxy derivatives (method B) in good yields. Conversion of 8 to the chloro analogue 4 was straightforward and also proceeded in reasonable yield (80%) (method C). Crown ether catalyzed direct nucleophilic displacement of the chlorine atom in 4 with "naked" halide[7] or "naked" acetate[8] to provide compounds 3 and 5–7 occurred under very mild conditions (method D). With the exception of "naked" bromide, greater than 80% yield was achieved. Incomplete reactions that resulted in impure products and thus the need for extensive purification may be the reason for poor yield in the "naked" bromide reaction. The direct displacement of chloride by "naked" fluoride, however, represents an excellent method for introducing fluorine into an organic molecule under mild conditions.

Pharmacological Results and Discussion

Antispasmodic activities of compounds synthesized were tested on isolated rat ileum preparations according to the method of Long and Chiou.[9] Briefly, an isolated portion of rat ileum was suspended in a tissue bath of aerated Tyrode solution and attached in an appropriate manner to a force-displacement transducer and connected to an ink-writing physiograph for recording experimental data. A dose–response experiment was carried out in which the contractions of the ileal tissue were measured with increasing concentrations of ACh in the tissue bath. The ability of each compound to block ACh-induced contractions of the ileum was then measured and a pA_2 value calculated according to the method of Ariens and van Rossum.[10] The ID_{50} value, the molar concentration of antagonist that inhibited 50% of a standard contraction response, was also calculated. These results are summarized in Table I and compared to those of atropine, which was also tested on isolated rat ileum preparations.



† Department of Medicinal Chemistry and Pharmacognosy.
‡ Department of Pharmacodynamics.

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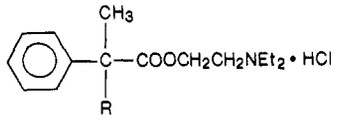
Table I. Antispasmodic Activity of Amino Esters of α-Methyltropic Acid



compd[a]	R	mp[b], °C	recrystn solvent	% yield	emp formula[c]	method[d] prepn	pharmacological data: pA_2[e]	pharmacological data: ID_{50}, M	pharmacological data: relative potency
(±)-1	CH_2OH	115–116.5[f]	EtOAc	81	$C_{16}H_{26}ClNO_3$	A	6.50 ± 0.06	3.14×10^{-7}	1
(−)-1a	CH_2OH	95–96.5[g]	EtOAc	96	$C_{16}H_{26}ClNO_3$[i]	A	7.79 ± 0.09	1.63×10^{-8}	19.26
(+)-1b	CH_2OH	93–95[h]	EtOAc	90	$C_{16}H_{26}ClNO_3$	A	5.94 ± 0.16	1.14×10^{-6}	0.27
(±)-2	CH_2OTs	103–105	EtOAc	94.5	$C_{23}H_{32}ClNO_5S$	B	7.41 ± 0.09	3.89×10^{-8}	8.07
(−)-2a	CH_2OTs	113–116	EtOAc	76	$C_{23}H_{32}ClNO_5S$	B	7.51 ± 0.15	3.10×10^{-8}	10.13
(+)-2b	CH_2OTs	115–116.5	EtOAc	74	$C_{23}H_{32}ClNO_5S$	B	6.77 ± 0.03	1.72×10^{-7}	1.83
3	CH_2F	120–121	EtOAc	80	$C_{16}H_{25}ClFNO_2$	D	6.52 ± 0.07	3.01×10^{-7}	1.04
4	CH_2Cl	110–111	EtOAc	80	$C_{16}H_{25}Cl_2NO_2$[j]	C	6.26 ± 0.15	5.54×10^{-7}	0.57
5	CH_2Br	87–89.5	EtOAc	16	$C_{16}H_{25}BrClNO_2$[k]	D	6.90 ± 0.07	1.27×10^{-7}	2.47
6	CH_2I	127–128.5	EtOAc	99	$C_{16}H_{25}ClINO_2$	D	7.41 ± 0.02	3.87×10^{-8}	8.11
7	CH_2OAc	100–102	EtOAc–cyclohexane	85	$C_{18}H_{28}ClNO_4$	D		3.94×10^{-7}[l]	0.80[l]
atropine							8.73 ± 0.04	1.84×10^{-9}	170

[a] Except where indicated all compounds are racemic. [b] Uncorrected. [c] All compounds had satisfactory C, H, and N microanalyses and were within 0.4% of theoretical values except when noted. All compounds exhibited IR, NMR, and MS spectra consistent with the assigned structures. [d] See Experimental Section. [e] pA_2 values were calculated according to Ariens and van Rossum[10] and mean values were obtained from linear regression analysis of two or more experiments at four or more dose levels. [f] Literature[11] 115.8–117.5 °C. [g] Literature[11] 93.5–95.5 °C. [h] Literature[11] 92.2–93.7 °C. [i] C: calcd, 60.86; found, 60.42. [j] C: calcd, 57.48; found, 57.06. [k] C: calcd, 50.80; found, 50.15. [l] This compound was tested earlier on isolated guinea pig according to the method described previously.[5] Under the same condition compounds 1, 3, and 4 have an ID_{50} value of 4.83×10^{-7}, 4.11×10^{-7}, and 1.17×10^{-6} M, respectively.

As shown in Table I, the antispasmodic activity of the amino ester of (*S*)-(−)-α-methyltropic acid (**1a**; $pA_2 = 7.79$) was found to be approximately 71 times greater than that of the *R*-(+) isomer, **1b** ($pA_2 = 5.94$). Although the antispasmodic activity on isolated rat ileum for these enantiomers has not been reported, our findings agree well with the reported values observed with isolated guinea pig ileum preparations (the log *K* values for **1a** and **1b** reported are 7.996 and 6.126, respectively, with an enantiomeric difference of 72 times). The observed difference between enantiomers may be explained on the basis of our postulation of a semirigid conformation via intramolecular hydrogen bonding.[5] For the *S*-(−) isomer, **1a**, intramolecular hydrogen bonding would yield a semirigid conformation directing the phenyl group to a site that specifically accommodates an aromatic ring. For the *R*-(+) isomer, **1b**, the semirigid conformation would only allow a methyl group to bind to this site. It is noteworthy that when the hydroxymethyl moiety of the α-methyltropate **1** is tosylated, the antispasmodic activity increased both in the case of racemate **2** and the less active *R*-(+) isomer **2b** while a 50% reduction in activity was observed for the *S*-(−) isomer **2a**. A possible explanation of this result is that, with the loss of intramolecular hydrogen bonding, *R*-(+) isomer **2b** could redirect its phenyl group to the site that prefers an aromatic ring. The bulky (tosyloxy)methyl group could then interact with the less restrictive hydrophobic region of the receptor. Furthermore, with the presence of two aryl groups in compounds **2**, the enantiomeric difference in potency is greatly reduced (i.e., from 71 to 5.5 times).

A similar situation was observed when the hydroxyl group of α-methyltropate **1** was replaced by a halogen. Little or no change in activity was found for the fluoro analogue **3**, while a slight decrease in potency was observed for the chloro analogue **4**. On the other hand, the bulky bromo derivative **5** and iodo derivative **6** provided compounds that were 2.5 and 8 times more active than the hydroxy compound **1**, respectively. This increase in the potency of **5** and **6** may be explained by a reorientation of the phenyl group to a site that is specific for an aromatic group and the bulky bromomethyl or iodomethyl group to the less restrictive hydrophobic region. The resolution of these halogenated analogues should provide further evidence for these conclusions; these are in progress.

Atropine and compounds **2** and **3** were further tested for their inhibitory activity against nonspecific antispasmodic activity on isolated rat ileum. A standard dose of 300 μg/mL of barium chloride, determined in a preliminary dose–response study, was used. Atropine, at a dose of 0.5 μg/mL (1.5×10^{-6} μM), caused only a 5.9 ± 1.6% reduction in ileum contraction. This indicates that its antispasmodic activity is indeed specific for the muscarinic receptor. Similar conclusions may be reached for compounds **2** and **3** by comparing their inhibitory activity against specific and nonspecific antispasmodic activity. At a dose level that completely blocked the effect of ACh-induced rat ileum contraction, both compounds produced partial reduction in barium chloride induced ileum contraction; i.e., compound **3**, at doses of 5.0 μg/mL (1.6×10^{-5} μM), 10.0 μg/mL (3.2×10^{-5} μM), and 20 μg/mL (6.4×10^{-5} μM) caused only 15.7 ± 3.4, 24.0 ± 2.5, and 35.7 ± 3.6% inhibitory activity while compound **2** at a dose of 3 μg/mL (6.4×10^{-6} μM) produced 33 ± 3.0% reduction in antispasmodic activity against barium chloride.

Furthermore, in the case of compound **2**, this blockade of ACh-induced ileum contraction can be easily removed by washing the tissue with fresh Tyrode solution. This indicates that the tosyloxy derivative **2** was unable to alkylate the possible hydrogen-bonding subsite of the receptor. The antispasmodic activities observed in the present study may suggest the absence of a hydrogen-bonding subsite. However, the question remains as to whether it may be situated in a very specific location under the large hydrophobic region. A more structurally rigid analogue may be needed to prove or disprove its presence in the muscarinic receptor.

Experimental Section

Chemistry. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 337 spectrophotometer. ^1H NMR spectra were obtained in $CDCl_3$ on a Varian T-60A spectrometer equipped with a Nicolet TT-7 Fourier transform accessory. Chemical shifts are reported in parts per

million (δ) downfield from tetramethylsilane as the internal standard. The abbreviations s, d, t, and m refer to singlet, doublet, triplet, and multiplet, respectively. Mass spectra were obtained at 70 eV with a Hitachi Perkin-Elmer RMU-D6 single-focusing mass spectrometer. Optical rotations were measured on a Perkin-Elmer polarimeter, Model 241, using a 1-dm tube. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, IL.

α-Methyltropic acid and its optical isomers were synthesized and resolved according to the method reported[6,12,13] with minor modification; i.e., ethyl 2-phenyl-2-(hydroxymethyl)-propanoate in 10% NaOH was stirred at room temperature for 3 h and then heated to 60 °C for 1.5 h. Pure **8** obtained (76.8% yield), mp 86–88 °C (lit.[6] mp 86 °C) was resolved into its enantiomers. **(*S*)-(−)-α-Methyltropic acid ((−)-8)**: mp 85.5–86.5 °C; $[\alpha]^{20}_D$ −26.6° (lit.[13] $[\alpha]^{20}_D$ −28.3°; mp 89–90 °C). **(*R*)-(+)-α-Methyltropic acid ((+)-8)**: mp 85–86.5 °C; $[\alpha]^{20}_D$ +26.6° (lit.[13] mp 88–90 °C; $[\alpha]^{20}_D$ +27°).

Preparation of 2-(Diethylamino)ethyl α-Methyltropate and Its Optical Analogues (1). Method A. (±)-α-Methyltropic acid (1.8 g, 0.01 mol) and 2-(diethylamino)ethyl chloride (freshly distilled, 1.5 g, 0.011 mol) in isopropyl alcohol (12 mL) were heated on a steam bath for 6 h. After cooling to room temperature, the mixture was filtered to remove a small amount of solid formed during the reaction. The filtrate was then concentrated to about half of its original volume under reduced pressure and diluted with water (50 mL). With ice bath cooling, the amino ester was liberated by dropwise addition of 10% NaOH (10 mL) and extracted into ether (3 × 50 mL). The ether layer was washed with water, dried (anhydrous $MgSO_4$) and evaporated to give a slightly yellowish oil (2.26 g, 81%): TLC, one spot R_f 0.59 (ether with 3% NH_4OH); ^1H NMR δ 0.95 (t, 6 H, CH_2CH_3), 1.57 (s, 3 H, CH_3), 2.50 (q, 4 H, CH_2CH_3), 2.63 (t, 2 H, J = 5.0 Hz, CH_2N), 3.50–4.30 (m, 4 H, CH_2OH, OCH_2), 4.61 (s, 1 H, OH), 7.23 (s, 5 H, Ar H); IR (neat) 3450 (br, OH) and 1750 cm^{-1} (C=O); MS, m/e 279 (M^+).

The hydrochloride salt of compound **1** was prepared by passing anhydrous hydrogen chloride gas into a dry ether solution of amino ester **1**. The white precipitate collected was recrystallized from ethyl acetate to give pure (±)-**1**, mp 115–116.5 °C (lit.[11] mp 115.8–117.5 °C). Anal. ($C_{16}H_{26}ClNO_3$) C, H, N.

Similarly prepared were the optical analogues **(−)-1a** and **(+)-1b** in 96% and 90% yields, respectively.

Preparation of 2-(Diethylamino)ethyl 2-Methyl-2-phenyl-3-(tosyloxy)propanoate and Its Optical Analogues (2). Method B. *p*-Toluenesulfonyl chloride (3.05 g, 0.016 mol) in anhydrous pyridine (50 mL) was cooled to 0 °C. With ice bath cooling and stirring, compound **1** (2.26 g, 0.008 mol) was added portionwise. The solution was allowed to stand in a refrigerator for 24 h and then at room temperature for an additional 5 h. The mixture was poured into ice water (200 mL) and extracted with ether (3 × 150 mL). The ether layer was washed repeatedly with water to remove pyridine, dried (anhydrous Na_2SO_4), and evaporated to give crude product **2** as an oil (3.34 g, 94.5%): TLC, one spot R_f 0.3 (ether with 3% NH_4OH); ^1H NMR δ 0.93 (t, 6 H CH_2CH_3), 1.67 (s, 3 H, CH_3), 2.30–2.70 (m, 9 H, NCH_2 and Ar CH_3), 4.03–4.63 (m, 4 H, OCH_2) and CH_2OSO_2), 7.30 (s, 5 H, Ar H), 7.20–7.82 (q, 4 H, Ar H); IR (neat) 1740 cm^{-1} (C=O); MS, m/e 433 (M^+).

The hydrochloride salt was obtained as described in method A above. After recrystallization from ethyl acetate, pure **2** was obtained, mp 103–105 °C. Anal. ($C_{23}H_{32}ClNO_5S$) C, H, N.

Similarly, optically active **(−)-2a** and **(+)-2b** were prepared from the corresponding optically active amino alcohols **(−)-1a** and **(+)-1b**, respectively.

Preparation of 2-(Diethylamino)ethyl 2-Methyl-2-phenyl-3-chloropropanoate (4). Method C. A mixture of α-methyltropic acid (**8**; 10 g, 0.05 mol) and thionyl chloride (80 mL) was heated on a steam bath for 3 h. The excess thionyl chloride was azeotropically removed with the aid of dry benzene under reduced pressure. Distillation of the residue yielded 7.0 g (58%) of pure **2-phenyl-2-(chloromethyl)propanoyl chloride**: bp 75–76 °C (0.05 mm); ^1H NMR ($CDCl_3$) δ 1.80 (s, 3 H, CH_3), 3.48 (s, 2 H, CH_2Cl), and 7.40 (s, 5 H, Ar H).

To a solution of 2-phenyl-2-(chloromethyl)propanoyl chloride (7.0 g, 0.03 mol) in dry benzene (50 mL) was added 2-(diethylamino)ethanol (7.5 g, freshly distilled, 0.06 mol) with stirring. The mixture was refluxed for 20 h on a steam bath and then allowed to cool to room temperature. It was extracted with 5% sodium bicarbonate (60 mL) and the benzene layer was separated. The alkaline solution was extracted twice with ether (50 mL). The ether extracts were combined with the benzene layer, washed with water, dried (anhydrous Na_2SO_4), and filtered. Removal of the solvent in vacuo gave a yellowish residue, 7.6 g (80%); the IR and NMR spectra indicated it was the expected product. The crude product was fractionally distilled to afford pure 2-(diethylamino)ethyl 3-chloro-2-methyl-2-phenylpropanoate (**4**), bp 115–118 °C (0.08 mm). The hydrochloride salt of the amino ester **4** was prepared by passing anhydrous hydrogen chloride gas into a dry ether solution of the amino ester. The white hydrochloride salt that precipitated was collected and recrystallized twice from ethyl acetate to yield a white crystalline solid: mp 110 °C; ^1H NMR ($CDCl_3$) δ 1.40 (t, 6 H, CH_2CH_3), 1.80 (s, 3 H, CH_3), 3.20 (m, 6 H, NCH_2), 3.38 (s, 2 H, CH_2Cl), 4.60 (t, 2 H, OCH_2), 7.30 (s, 5 H, Ar H); MS, m/e 297 (M^+ − HCl). Anal. ($C_{16}H_{25}Cl_2NO_2$) C, H, N.

Preparation of 2-(Diethylamino)ethyl 3-Fluoro-2-methyl-2-phenylpropanoate (3). Method D. Crown Ether Catalyzed Direct Displacement Reaction. Dicyclohexyl-18-crown-6 (0.745 g) was dissolved in acetonitrile in a 10-mL volumetric flask. The prepared solution was then poured directly over solid, dry potassium fluoride, (465 mg, 0.008 mol, freshly purified and dried) in a 25-mL round-bottomed flask. The heterogeneous solution was stirred at room temperature for 30 min.

In a 25-mL round-bottomed flask equipped with a reflux condenser and drying tube was placed 1.2 g (0.004 mol) of the amino ester **4** and 10 mL of the freshly prepared crown ether–potassium fluoride solution. The mixture was heated to reflux with vigorous stirring for 288 h. The reaction mixture was then allowed to cool to room temperature, filtered, and concentrated to about one-third of its original volume. About 20 mL of distilled water was added and the mixture extracted several times with chloroform. The combined chloroform extracts were dried (anhydrous $MgSO_4$), filtered, and evaporated to dryness under reduced pressure. The residue was then purified by dissolving in 5% aqueous HCl solution (25 mL) and washing with ether to remove crown ether. It was then neutralized with solid sodium bicarbonate to liberate the free amine. This was extracted with chloroform and the organic layer was washed with water. This purification step was repeated until there was no more crown ether complex as indicated by NMR analysis. The chloroform extracts were dried (anhydrous $MgSO_4$), filtered, and evaporated to dryness under reduced pressure. This gave 0.9 g (80%) of **3** as a yellowish oil: ^1H NMR ($CDCl_3$) δ 1.0 (m, 6 H, NCH_2CH_3), 2.50 (m, 6 H, NCH_2), 1.75 (s, 3 H, CH_3), 4.0 (m, 4 H, CH_2O and CH_2F), 7.30 (s, 5 H, Ar H). The hydrochloride of the amino ester **3**, after it was recrystallized from ethyl acetate, melted at 120–121 °C; MS, m/e 280 (M^+ − HCl). Anal. ($C_{16}H_{25}ClFNO_2$) C, H, N.

Similarly, compounds **5–7** were prepared in 16%, 99%, and 85% yield, respectively, from **4** and the corresponding "naked" anions.

Pharmacology. Materials and Methods. Male Sprague–Dawley rats weighing 250–300 g were purchased from King Animal Laboratories, Oregon, WI. Each animal used in these experiments were fasted overnight and sacrificed by cervical dislocation. A Narco-Biosystems four-channel ink-writing physiograph (Table Model No. DMP-4A) and a force-displacement transducer (Narco-Biosystems F-1000) were used to record smooth muscle contractions. All pharmacological testing procedures on isolated rat ileum preparations were as described previously.[5]

Nonspecific Antispasmodic Activity on Isolated Rat Ileum. The nonspecific antispasmodic activity of atropine and compounds **2** and **3** was determined on strips of rat ileum in a tissue bath as described previously.[5] A standard dose of barium chloride (300 μg/mL) as predetermined from a preliminary dose–response experiment was introduced into the tissue bath to induce smooth muscle contractions. After the maximal con-

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traction had been established, barium chloride was removed by repeated washing with fresh Tyrode solution. Several washes were necessary to return tissue contraction back to the base line. After the tissue had stabilized, a given dose of the test compound was added and allowed 10 min for equilibration. Without washing out the test compound, an identical dose of barium chloride (300 μg/mL) was added, and contractions were recorded. The difference between smooth muscle contraction induced by barium chloride before and after the administration of the test compound was calculated in terms of percent inhibition.

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Registry No. (±)-**1**, 105401-26-1; (±)-**1**·HCl, 50700-19-1; (−)-**1a**, 99545-92-3; (−)-**1a**·HCl, 50700-18-0; (+)-**1b**, 99553-27-2; (+)-**1b**·HCl, 50700-17-9; (±)-**2**, 105401-27-2; (±)-**2**·HCl, 105401-33-0; (−)-**2a**, 105497-78-7; (−)-**2a**·HCl, 105497-79-8; (+)-**2b**, 105498-87-1; (+)-**2b**·HCl, 105498-88-2; (±)-**3**, 105401-28-3; (±)-**3**·HCl, 105401-34-1; (±)-**4**, 105401-29-4; (±)-**4**·HCl, 105401-35-2; (±)-**5**, 105401-30-7; (±)-**5**·HCl, 105401-36-3; (±)-**6**, 105401-31-8; (±)-**6**·HCl, 105401-37-4; (±)-**7**, 105401-32-9; (±)-**7**·HCl, 105401-38-5; (±)-**8**, 31917-13-2; (+)-**8**, 28968-34-5; (−)-**8**, 59492-59-0; (±)-$C_6H_5C(CH_2OH)(CH_3)CO_2Et$, 70397-73-8; (±)-$C_6H_5C(CH_2Cl)(CH_3)COCl$, 105401-39-6; $Et_2N(CH_2)_2OH$, 110-73-6; $Et_2N(CH_2)_2Cl$, 100-35-6.

The Mechanism of Activation of 4-Hydroxycyclophosphamide

Richard F. Borch* and Jo Ann Millard

Department of Pharmacology and the Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received June 9, 1986*

4-Hydroxycyclophosphamide (**2**/**3**) of unknown stereochemistry is the initial metabolite formed after administration of cyclophosphamide (**1**). Ultimate conversion to the cytotoxic metabolite phosphoramide mustard (**6**) is initiated by ring opening of 4-hydroxycyclophosphamide to produce aldophosphamide (**4**). The ring-opening reaction and subsequent equilibration of **2**–**4** are subject to general-acid catalysis, and the equilibrium composition is independent of buffer structure and pH. In contrast, formation of **6** from **4** proceeds by general-base-catalyzed β-elimination. *trans*-4-Hydroxycyclophosphamide undergoes ring opening ca. 4 times faster than the cis isomer, and cyclization of **4** favors the trans isomer by a factor of ca. 3 over the cis isomer. The rapid equilibration of **2**–**5** and the absence of elimination to give **6** at pH ~5 provides a convenient method to prepare a stable equilibrium mixture of activated cyclophosphamide metabolites suitable for in vitro use.

Cyclophosphamide (**1**) and its analogues are of considerable interest because of their therapeutic efficacy in the treatment of cancer and for the complexity of the activation mechanism that they undergo. The overall activation process has been reviewed;[1-3] more recently, we[4] and others[5] have described the mechanistic details of this process. Activation is initiated by hepatic cytochrome P-450 oxidation, and the resulting 4-hydroxy compounds **2** and/or **3** (see Scheme I) establish an equilibrium with aldophosphamide (**4**) and its hydrate **5**. General-base-catalyzed elimination of phosphoramide mustard from **4** is the rate-limiting activation step under physiologic conditions.[4,5,7] Enzymes are not required for catalysis after the initial hydroxylation. Intermediates **2**–**5** can be oxidized further to produce inactive metabolites. Although the aldehyde is clearly the pivotal intermediate in the activation process, it represents <5% of the metabolite mixture at equilibrium. Imine **9** has been identified as a transient intermediate in the enzymatic activation of **1**,[8] in the hydrolysis of 4-hydroperoxycyclophosphamide,[9] and in the activation of the 4-alkylthio-substituted analogue mafosfamide.[10] Compounds substituted with thiol groups at the 4-position show clinical promise as "preactivated" analogues[11] and may have significance as in vivo metabolites of **1**. We describe here our studies of the **2**–**5** equilibrium that demonstrate the importance of both acid- and base-catalyzed processes to cyclophosphamide activation; the chemistry of mafosfamide is presented in the accompanying paper.[10]

Table I. Equilibrium Constants for *cis*-**2** and *trans*-4-Hydroxycyclophosphamide (**3**) and Aldophosphamide + Hydrate (**4** + **5**)[a]

buffer (pH)	2/4 + 5	3/4 + 5	2/3
acetate (4.3–5.3)	2.28 ± 0.20	1.68 ± 0.07	1.36 ± 0.12
cacodylate (5.4–7.3)	2.55 ± 0.36	1.70 ± 0.19	1.50 ± 0.19
citrate (5.0–6.3)	2.61 ± 0.19	1.83 ± 0.13	1.43 ± 0.14
phosphate (5.3–7.0)	2.68 ± 0.24	1.82 ± 0.15	1.47 ± 0.14
mean	2.53 ± 0.17	1.76 ± 0.08	1.44 ± 0.06

[a] Mean ± SEM for at least six time points per experiment and at least four experiments at different pH values for each buffer.

Results and Discussion

cis-4-Hydroperoxycyclophosphamide (**13**) was synthesized in 40–50% reproducible yields according to the published procedure.[12] We have found the yields to be more consistent than with the method involving direct ozonolysis of cyclophosphamide.[9,13] *cis*-4-Hydroxycyclophosphamide

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